

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 47/48		(11) International Publication Number: WO 92/11872
A1		(43) International Publication Date: 23 July 1992 (23.07.92)
(21) International Application Number: PCT/US92/00101 (22) International Filing Date: 2 January 1992 (02.01.92) (30) Priority data: 637,074 3 January 1991 (03.01.91) US (71) Applicants: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US). THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Washington, DC 20201 (US). (72) Inventors: CASSELLS, Ward ; 3509 "T" Street, N.W., Washington, DC 20007 (US). LAPPI, Douglas, A. ; 12842 Caminito de las Olas, Del Mar, CA 92014 (US). BAIRD, J., Andrew ; 5039 Via Papel, San Diego, CA 92122 (US).		(74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), RU, SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MITOTOXIN FOR TREATMENT OF VASCULAR INJURY		
(57) Abstract <p>Patients suffering from vascular injury as a result of balloon catheterization or the like are treated with medicaments containing conjugates comprising a ligand, such as bFGF (or another FGF polypeptide), and a cytotoxic agent. The cytotoxic agent can be a ribosome-inactivating protein (RIP), such as saporin, and it is attached to the ligand through a chemical bond or prepared as a recombinant DNA chimera. The medicament containing the conjugate is administered IV to patients after they have been treated for atherosclerosis in a manner which commonly results in vascular injury, particularly to the intima, and is effective in preventing restenosis. The ligand binds to and activates the high-affinity bFGF receptors which are surprisingly expressed on smooth muscle cells in the lumen of the blood vessels allowing the cytotoxic agent to enter the target cells and ultimately destroy the undesired proliferating cells.</p>		

BEST AVAILABLE COPY

ATTORNEY DOCKET NUMBER: 10177-191-999
SERIAL NUMBER: 10/603,115
REFERENCE: B95

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : A61K 47/48</p>	<p>A1</p>	<p>(11) International Publication Number: WO 92/11872 (43) International Publication Date: 23 July 1992 (23.07.92)</p>
<p>(21) International Application Number: PCT/US92/00101 (22) International Filing Date: 2 January 1992 (02.01.92) (30) Priority data: 637,074 3 January 1991 (03.01.91) US (71) Applicants: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US); THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Washington, DC 20201 (US). (72) Inventors: CASSCELLS, Ward ; 3509 "T" Street, N.W., Washington, DC 20007 (US). LAPPI, Douglas, A. ; 12842 Caminito de las Olas, Del Mar, CA 92014 (US). BAIRD, J., Andrew ; 5039 Via Papel, San Diego, CA 92122 (US).</p>		<p>(74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), RU, SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: MITOTOXIN FOR TREATMENT OF VASCULAR INJURY</p> <p>(57) Abstract</p> <p>Patients suffering from vascular injury as a result of balloon catheterization or the like are treated with medicaments containing conjugates comprising a ligand, such as bFGF (or another FGF polypeptide), and a cytotoxic agent. The cytotoxic agent can be a ribosome-inactivating protein (RIP), such as saporin, and it is attached to the ligand through a chemical bond or prepared as a recombinant DNA chimera. The medicament containing the conjugate is administered IV to patients after they have been treated for atherosclerosis in a manner which commonly results in vascular injury, particularly to the intima, and is effective in preventing restenosis. The ligand binds to and activates the high-affinity bFGF receptors which are surprisingly expressed on smooth muscle cells in the lumen of the blood vessels allowing the cytotoxic agent to enter the target cells and ultimately destroy the undesired proliferating cells.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroun	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

Mitotoxin for treatment of vascular injury

This invention generally relates to the use of
5 fibroblast growth factor ligands conjugated to cytotoxic
agents in a manner to inhibit undesired cell
proliferation, and more specifically, to the treatment of
patients who have experienced vascular injury or are
otherwise experiencing the proliferation of cells that
10 express functional high-affinity bFGF receptors by
administering a mitotoxin that includes a ligand such as
FGF.

Background of the Invention

15

Despite declining fatality rates, athero-
sclerosis remains the leading killer in industrialized
nations. Attempts to prevent or to reverse athero-
sclerosis, and rapid smooth muscle proliferation which
20 contributes importantly to pulmonary hypertension,
transplant rejection, and restenosis after angioplasty,
or bypass grafting, include the use of antihypertensive
and cholesterol-lowering agents, fish oils,
corticosteroids, cyclosporin A, heparin and non-
25 anticoagulant heparin fragments, inhibitors of
angiotensin-converting enzyme, calcium antagonists,
aspirin, prostacyclin and other modulators of platelet
and smooth muscle eicosanoid metabolism, colchicine,
terbinafine, triazolopyrimidine, analogues of
30 somatostatin, anti-neoplastic agents, seeding with
endothelial cells, and devices (intended to produce less
vascular trauma than balloon angioplasty), such as laser
angioplasty and atherectomy. Preliminary results in
experimental animals and in humans show promise for some
35 of these therapies; however, most of these will require
sustained or local application.

-2-

Basic fibroblast growth factor (bFGF) is a protein which has a molecular weight of approximately 16 kD, is acid- and temperature-sensitive and has a high isoelectric point. A structurally related protein, acidic FGF (aFGF), has an acidic isoelectric point. FGFs exhibit a mitogenic effect on a wide variety of mesenchymal, endocrine and neural cells. Of particular interest is their stimulatory effect on collateral vascularization and angiogenesis. Such mitogenic effects have stimulated considerable interest in FGFs as potential therapeutic agents for wound healing, nerve regeneration and cartilage repair, for example.

New capillary growth takes place by a series of sequential steps beginning with the dissolution of the capillary basement membrane. Microvascular endothelial cells stimulated by angiogenic substances, such as bFGF, in vitro secrete collagenase, plasminogen activator, and stromelysin which degrade the basement membrane and allow endothelial cells to migrate toward the angiogenic stimulus. After migrating, the endothelial cells proliferate, develop sprouts, form capillary-like hollow tubules, and finally link tubules into capillary loops.

Many cells that respond to basic FGF have been shown to possess specific receptors on the cell surface membranes. The receptor proteins appear to be single chain polypeptides with molecular weights ranging from 110 to 150 kD, depending on cell type. These receptor proteins bind basic FGF with high affinity ($K_d = 10^{-8}$ to 10^{-9} M), and receptor numbers often range from 2000 to 80,000 per cell. Such receptors have been purified from chicken embryo and from rat brain, using a combination of lectin and ligand affinity chromatography and are associated with tyrosine kinase activity, see Imamura et al., B.B.R.C. 155, 583-590 (1989); Huang and Huang, J. Biol. Chem., 261, 9568-9571 (1986).

-3-

On baby hamster kidney cells (BHK), two basic FGF receptors with estimated molecular weights of 110 and 130 kD have been reported in Neufeld et al., J. Biol. Chem., 260, 13860-13868 (1985) and Neufeld et al., J. Biol. Chem., 261, 5631-5637 (1986). Although both receptor proteins bind basic FGF and acidic FGF, it appears that the larger receptor protein binds bFGF preferentially and is sometimes referred to as the "high affinity" bFGF receptor; the smaller receptor has

5
10 somewhat greater affinity for acidic FGF.

The feasibility of using receptor-specific ligands to transport toxins into cells has recently been demonstrated. The strategy, originally applied in immunotherapy by conjugating toxins to monoclonal

15 antibodies (see Blakey et al., Cancer Research, 48, 7072-7078 (1988)), has recently been pursued by coupling toxins with classic endocrine hormones, such as CRF and TRF, with cytokines such as EGF and TGF α and with lymphokines such as interleukin-2. U.S. Patent No.

20 4,468,382 to Bacha et al. shows cytotoxic conjugates wherein the hormone TRH is linked to the toxin CRM 45 by a disulfide bond with a histidine residue to produce a toxic hybrid protein alleged to be useful in the treatment of certain tumors.

25 Atherosclerosis, sometimes referred to as arteriosclerosis, is a result of the development of an intimal lesion and the subsequent narrowing of the vessel lumen. Commonly, atherosclerosis originally appears as a result of the buildup of plaque which lines the interior

30 of blood vessels, particularly the arteries. Whereas bypass surgery is sometimes employed to replace such clogged arteries, in recent years, a number of surgical procedures have been developed so as to interarterially remove such plaque, often by balloon catheterization or

35 other such treatments in which the plaque is either compressed against or scraped away from the interior

-4-

surface of the artery. Not infrequently, the patient so treated finds a recurrence of such narrowing of the vessel lumen in a relatively short period thereafter, generally referred to as restenosis, requiring a
5 repetition of the surgical procedure to again remove the increasing blockage. The need exists for preventing such recurrence in patients who have been treated for atherosclerosis.

10 Summary of the Invention

Basic fibroblast growth factor (bFGF) has never been implicated in atherosclerosis or in neointimal hyperplasia in response to vascular injury; however, it
15 has been surprisingly found that the administration of a conjugate of a ligand, such as bFGF, and a cytotoxic agent can effectively prevent neointimal hyperplasia. Although bFGF mRNA, FGF receptor mRNA and bFGF protein are nearly undetectable in normal rat vessels, it has now
20 been found that they are expressed after injury, particularly after dilation or crush injury, which can cause neointimal hyperplasia and result in restenosis.

By coupling a ligand, such as basic FGF, to saporin-6 (SAP), a ribosome-inactivating protein (RIP)
25 isolated from the seeds of the plant Saponaria officinalis, a powerful mitotoxin (FGF-SAP), is produced, i.e., a cytotoxic molecule targeted to specific cells by a mitogen. Certain conjugates like these are disclosed in International Application published November 1, 1990
30 as WO 90/12597. It has been found that after balloon catheter or other treatment of arteries, denudation of the interior wall of the vessel occurs, with the endothelial cells which constitute the lining of the blood vessels being removed in multiple different
35 locations. As a result of this removal, the smooth muscle cells, which are normally located exterior of the

-5-

endothelial cells and form the blood vessel structure, begin to grow and multiply. As a consequence, there is a tendency for these smooth muscle cells to express functional high-affinity bFGF receptors and to proliferate so as to again fill the interior of the blood vessel, much in the same manner which it was previously clogged by the buildup of plaque. It has now been discovered that such undesirable growth of these proliferating cells, for example, migrating smooth muscle cells in the interior of blood vessels which can result in restenosis, can be inhibited by the administration of conjugates of appropriate ligands and cytotoxic agents, such as FGF-SAP.

Administration of a medicament containing conjugates of bFGF-saporin in a manner so as to reach the region where such cells expressing these receptors are proliferating has been found to result in the invasion of these cells and the inhibition of both protein and DNA synthesis, killing the cells; and this has been demonstrated with proliferating smooth muscle cells both in vitro and in vivo. Treatment is effected by administering a therapeutically effective amount of a medicament containing the conjugate in a physiologically acceptable carrier or recipient, in a manner so that the conjugate reaches regions in a human or other mammal where the cytotoxic agents then inhibit the proliferation of the target cells. Although a single dose inhibits neointimal proliferation, IV administration over a period of time is preferred.

Detailed Description of the Preferred Embodiments

A key event in arteriosclerosis is the development of an intimal lesion with subsequent narrowing of the vessel lumen. Smooth muscle cell replication occurs after balloon catheter denudation of

-6-

the rat carotid artery, and a comparison of two methods of experimental arterial injury has shown that smooth muscle replication appears to correlate with medial damage rather than with complete endothelial loss. This suggests the possibility that injury-induced release of endogenous mitogens, such as basic fibroblast growth factor (bFGF) may play a pivotal role in the subsequent responses of the vascular wall. bFGF is known to be synthesized by both endothelial and smooth muscle cells (SMCs) and is thought to be stored in the subendothelial matrix, and in some instances, this growth factor is released from cells after injury. Further investigation has now shown that bFGF is a potent mitogen for migrating SMCs of injured arteries, that continuous administration of bFGF could greatly increase arterial lesion size, and that administration of a conjugate of a ligand, such as bFGF, and a cytotoxic agent can prevent such undesirable intimal growths in injured arteries in Sprague-Dawley rats.

Testing has now shown that basic FGF infused intraarterially into animals immediately after balloon catheter denudation or after denudation of endothelium by a less traumatic technique using a filament loop (which removes all the endothelium but does not significantly damage the underlying tunica media) causes a significant increase in smooth muscle cell proliferation.

Normally, 6 weeks after balloon injury, a substantial intimal thickening has formed, yet the smooth muscle replication is low. bFGF infused 6 weeks after denudation showed that replication of the intimal smooth muscle cells from the deendothelialized segment of the carotid artery was significantly increased by the administration of bFGF, with the intimal cells in close proximity to the lumen showing the highest proliferation rate.

-7-

After first demonstrating that bFGF was mitogenic for both medial and intimal smooth muscle cells following injury, prolonged bFGF administration was carried out to evaluate its effect on intimal thickening formation. It was shown that the intimal area of bFGF-treated animals was significantly increased, i.e. it was approximately twice as large as the intima from control animals.

It has now been found that treatment with conjugates of a cytotoxic agent and a ligand, such as bFGF, or another FGF polypeptide reactive with an FGF receptor, will effectively inhibit growth and proliferation of certain cells which express functional high-affinity bFGF receptors without substantial undesirable side effects. By inhibiting proliferation of migrating smooth muscle cells (SMCs), it is possible to prevent the undesirable growth and ultimate clogging which occurs following vascular injury, and which is generally referred to as restenosis. The conjugates employed comprise a ligand, such as basic FGF or another FGF polypeptide (or equivalent non-peptide), which is reactive with the high-affinity FGF receptor and a cytotoxic agent, particularly a ribosome-inactivating protein (RIP), such as saporin--although other cytotoxic agents can also be advantageously used. The cytotoxic agent can be attached to the ligand through a chemical bond; alternatively, the composition can be prepared as a chimera, using recombinant DNA techniques. In either case, the conjugate molecule is designed and produced in such a way that the receptor-binding epitope of the ligand moiety of the complex is left available for recognition by the high-affinity FGF receptor.

In addition to basic FGF (bFGF) and acidic FGF (aFGF), there are known to be a number of other proteins exhibiting basic FGF mitogenic activity mediated through binding to an FGF receptor. Moreover, it is now possible

-8-

to design non-peptide equivalents of the polypeptide bFGF which will also bind to and activate the bFGF receptor. Mammalian basic FGF is a 146-residue peptide having a molecular weight of about 16 kD, and a pI of about 9.6, which may have an amino terminal extension. Other FGF proteins in addition to aFGF include HST, INT/2, FGF-5, FGF-6, and KGF(FGF-7), see Baird et al., Brit. Med. Bull., 45, 438-452 (1989). All induce mitogenic activity in a wide variety of normal diploid mesoderm-derived and neural crest-derived cells. A test of such "FGF mitogenic activity" is the ability to stimulate proliferation of cultured bovine aortic endothelial cells, as described in Gospodarowicz et al., J. Biol. Chem., 257, 12266-12278 (1982) and Gospodarowicz et al., P.N.A.S., 73, 4120-4124 (1976). The term FGF is broadly used herein to include proteins having amino acid sequences found in mammalian hosts, as well as modified sequences, i.e. having amino acid substitutions, deletions, insertions or additions, which still exhibit mitogenic activity mediated through binding to the high-affinity FGF receptor. For example, purified preparations of native bFGF and aFGF are frequently observed to include several molecular forms of the mitogens, such as shortened fragments; moreover, differences in amino acid sequences occur in FGFs from different species. The term FGF is thus intended to encompass proteins isolated from natural sources, polypeptides made synthetically, either by recombinant means or by chemical synthesis, as well as chemically designed equivalent non-peptides reactive with the high-affinity bFGF receptor.

The amino acid sequence of an exemplary mammalian bFGF obtained from bovine pituitary tissue is reported in Esch et al., P.N.A.S., 82, 6507-6511 (1985); it is also set forth in U.S. Patent No. 4,956,455, issued September 11, 1990. The term "bFGF" should be generally

-9-

understood to refer to proteins or polypeptides having substantially the same amino acid sequence and mitogenic activity as that of bovine bFGF or human bFGF. cDNAs encoding human aFGF, see Jaye et al., Science, 233, 541-545 (1986); bovine bFGF, see Abraham et al., Science, 233, 545-548 (1986), human bFGF, see Abraham et al., EMBO J., 5, 2523-2528 (1986), and Abraham et al., Quant. Biol., 51, 657-668 (1986), and rat bFGF, see Shimasaki et al., B.B.R.C. (1988) and Kurokawa et al., Nucleic Acids Res., 16, 5201 (1988) have been cloned and sequenced; they predict the existence of proteins identical, or very highly homologous to bovine bFGF and aFGF found by protein sequencing.

As used herein, the term "FGF receptor" is used to refer to receptors, particularly the high-affinity receptors, which are able to bind basic FGF and transport it into the cell. Included among these are the receptors described in T. Imamura, B.B.R.C., 155, 583-590 (1988) and in Moscatelli, J. Cell. Physiol., 131, 123-130 (1987). As used herein, the term "ligand reactive with the FGF receptor" refers to any polypeptide or non-peptide equivalent which is capable of binding an FGF receptor and of being transported into the cell thereby.

Basic FGF is commercially available, for example, from Amgen (Thousand Oaks, CA) and from Amersham International. It can also be obtained from a variety of tissue types of mammals via methods of purification using reverse-phase high performance liquid chromatography (RP-HPLC) and/or heparin-Sepharose affinity chromatography. In addition, bFGF can be synthesized using recombinant methods. Expression of a recombinant protein in yeast and E. coli is described in Barr et al., J. Biol. Chem., 263, 16471-16478 (1988) and in U.S. Patent No. 4,956,455.

Such an FGF-cytotoxic agent conjugate can be purified on an immunoaffinity column containing immobilized heparin--for example, columns of heparin-

-10-

Sepharose or heparin-agarose. The bound conjugate can be eluted with a salt gradient, such as NaCl, and elutes between 1 and 3 M NaCl.

The ligand, e.g. bFGF, conjugated to a
5 cytotoxic agent, is used to target the cytotoxic agent to specific cells of interest. As used herein, the term cytotoxic agent refers to a molecule capable of inhibiting cell function. The term includes agents which are only toxic when transported into the cell and also
10 those whose toxic effect is mediated at the cell surface. A variety of cytotoxic agents can be used, particularly those which inhibit protein synthesis. As one example, bFGF is combined with a ribosome-inactivating protein (RIP) such as, for example, saporin-6 (SAP) or other SAP
15 derivatives. SAP is a potent RIP which is isolated from the seeds of the plant Saponaria officinalis, see Stirpe et al., Biochem J., 216, 617-625 (1983). Other appropriate cytotoxic agents include, but are not limited to, ricin, ricin A chain, gelonin, diphtheria toxin,
20 diphtheria toxin A chain, pokeweed antiviral protein (PAP), and Pseudomonas exotoxin. Alternatively, it may be feasible to use a drug as the cytotoxic agent; examples of such drugs include anthracyclines, such as the daunomycins (including daunorubicin and doxorubicin)
25 and methotrexate and its analogs.

A ligand, such as bFGF, is suitably conjugated to a protein cytotoxic agent by known chemical reactions, such as through derivatization with a reactive
30 sulfhydryl-containing moiety, such as SPDP, or via a cross-linking agent, such as glutaraldehyde or carbodiimide. For example, the cytotoxic agent may be derivatized with a reactive sulfhydryl containing agent, such as N-succinimidyl-3(2-pyridyldithio)propionate, before bFGF is added and mixed therewith. The bFGF
35 conjugate can be separated from the unreacted products on a suitable column. Alternatively, bFGF can be conjugated

-11-

to a drug, such as 14-bromo doxorubicin through the sugar moiety, as by the cis-aconitase method, see Shen and Riser, B.B.R.C., 102, 1048 (1981).

Alternatively, it may be possible to prepare
5 chimeric FGF-conjugates by recombinant methods. Such methods as applied to conjugates of IL-2 or TGF α are described in Chaudhary et al., P.N.A.S., 84, 4538-4542 (1987) and in Lorberman-Galski et al., P.N.A.S., 85, 1922-1926 (1988). See also Maniatis et al., Molecular
10 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982).

As hereinbefore indicated, it was unexpectedly found that vascular injury led to expression of high-affinity FGF receptors on cells such as migrating SMCs.
15 Indeed, within 24 hours of balloon dilation and deendothelialization of the rat carotid artery, or injury of the lung by endotoxin, Northern analysis revealed expression of 4.2 and 2.7 kb transcripts that hybridized with a cDNA for the FGF receptor gene. In situ
20 hybridization also revealed hybridization of an anti-sense cRNA for the FGF receptor gene. Moreover, autoradiograms of histologic sections revealed high affinity binding of bFGF (i.e., not displaceable by heparin infusion) to neo-intimal and medial migrating
25 smooth muscle cells of injured vessels but not to quiescent smooth muscle cells of normal vessels; this binding was noted within 24 hours of injury. It was competed by excess bFGF; however, heat-denatured tracer was not bound. It is felt that the majority of such
30 binding must have been to smooth muscle cells based on the light microscopic morphology and on the fact that leukocytes constitute a small minority of the medial cell population at this time.

The proliferating smooth muscle cells, as well
35 as monocyte/macrophages, also revealed immunocytochemical reactivity with peptide antisera raised against intra-

-12-

and extra-cellular domains of the mouse FGF receptor clone. Injured vessels also revealed increased bFGF expression by specific immunocytochemistry using several monoclonal and polyclonal antibodies (which gave no stain
5 after adsorption with excess ligand) and by in situ hybridization. Thus, injury, like culturing, induces expression of both bFGF and an FGF receptor.

Thymidine incorporation by medial smooth muscle cells began by 24 hours and was maximal at 48-96 hours.
10 Areas of thrombosis and leukocyte attachment and infiltration were noted from 24-72 hours. Subsequently, cells migrated across damaged internal elastic laminae and proliferated in the neointima. DNA synthesis declined gradually but had not normalized by 14 days. A
15 neointima was noted by 7 days and by 14 days it was 6-20 cells thick.

Although saporin itself is not believed to be toxic to most cells, if saporin is conjugated to bFGF, it is taken up by cells that express the high-affinity bFGF
20 receptor and becomes a potent toxin that causes cell death. It was anticipated that this mitotoxin would compete for receptor binding of bFGF in SMCs and mediate cell death after internalization, resulting in widespread cell death after administration of this mitotoxin. Six
25 weeks following denuding of endothelium with a balloon catheter from one artery of a group of rats, equimolar concentrations of bFGF conjugated to saporin (bFGF-SAP) or of unconjugated bFGF and saporin were infused intraarterially immediately prior to balloon injury.
30 Loss of medial SMCs in the denuded arteries was observed.

It was found that bFGF conjugated to saporin, when infused in balloon catheter-denuded vessels caused a significant decrease in the number of migrating SMCs in such arteries, reflective of widespread cell death.
35 Saporin not conjugated to bFGF was found not to be toxic to such SMCs because saporin by itself does not appear to

-13-

be readily transported into such cells. When bound to bFGF, however, saporin is carried into these cells via the high-affinity bFGF receptor-mediated pathway leading to cell death. It is believed that an intact endothelium
5 may act as a permeability barrier to both bFGF and the bFGF-saporin conjugate preventing their contact with the underlying SMCs.

To prove that such smooth muscle cell proliferation could be inhibited by bFGF-saporin, rabbit
10 aortic SMC from 8th passage were plated at 30,000 cells/cm² in 10% FBS in leucine-free M199. At 20 hours, cells received 0.01-10nM bFGF-saporin conjugate. At 14, 18 or 24 hours, cells were pulsed for 2 hours with 1μCi/0.5ml 3H-leucine, then chased with leucine-containing M199 for
15 2 hours and counted by liquid scintillation. This experiment was repeated once, with similar results, using rat aortic SMC.

In 10% serum, 10nM bFGF-saporin inhibited protein synthesis by 24 hours. Cell death began by 24-48
20 hours. Cells were killed by as little as 1 hour exposure to bFGF-saporin followed by a heparin wash to remove bFGF-saporin from heparin sulfates on the cell surface. Saporin alone inhibited cell growth only at concentrations of 0.1-1.0 μM.

25 By 96 hours after a single exposure to 10 nM bFGF-saporin, only 10% of cells were viable as indicated by exclusion of trypan blue. Since even quiescent cells require protein synthesis, the survival of these cells may be due to resistance to saporin in some cells or a
30 failure of bFGF-saporin to enter the cells. Smooth muscle cells appear to have few or no FGF receptors if quiescent. After balloon injury of the carotid artery, local application of bFGF-saporin kills proliferating smooth muscle cells, and intravenous bFGF-saporin
35 inhibits their migration and/or proliferation.

-14-

In rats subjected 24 hours earlier to balloon injury of the carotid artery, 1 or 10 μ g bFGF-saporin (instilled into a temporary pouch made by occluding the artery proximally and distally for 15 minutes) resulted in the death of most medial smooth muscle cells, and 75% fewer neointimal cells (compared to controls) were detected 14 days after injury. In the arteries of treated rats, there was as much thrombus as in saline controls, and even more inflammation. Since macrophages express multiple growth factors, and thrombin and fibrin are mitogenic for smooth muscle cells, the abundance of these processes suggests that bFGF-saporin is not acting indirectly, e.g., by a (hypothetical) anti-inflammatory or anti-thrombotic action. It appears that bFGF-saporin directly killed migrating smooth muscle cells and that this injury accentuated the thrombotic and inflammatory responses to the balloon injury.

Rats given intravenous bFGF-saporin as a single 25 μ g dose 24 hours after balloon injury developed 24% less neointimal proliferation at 14 days as measured by surface area. In this model, there was almost no evidence of thrombosis at 14 days, and inflammation was much less marked than in the rats treated by local infusion. In fact, the absence of necrosis in the rats treated with bFGF-saporin suggests that some of the effect is due to an inhibition of proliferation and/or migration rather than cell killing.

The effect of bFGF-SAP upon pulmonary vascular injury, thrombosis, inflammation and smooth muscle proliferation caused by endotoxin was also investigated. Endotoxin causes death of some endothelial cells while others are stimulated to produce leukocyte adhesion molecules, pro-coagulant factors and cytokines mitogenic for smooth muscle cells, such as interleukin 1, PBGF-B chain, TNF and endothelin. Rats were given 1 μ g lipopolysaccharide intraperitoneally (2×10^4 endotoxin

-15-

units/ μ g, Ribi Immunochem, Hamilton MT), followed by 24 hours by 10 μ g saporin or bFGF-saporin, IV. Following sacrifice on day 7, there were fewer foci of injury in those treated with the conjugate, and many vessels in fact appeared thinned. Intravascular thrombi in rats treated with saporin contained smooth muscle cells, while those in rats treated with bFGF-saporin had none.

Present therapies for the treatment of atherosclerosis, for example balloon catheterization, inherently result in some injury to the endothelial lining of blood vessel walls, thereafter requiring follow-on local application. Although it can be administered locally, a medicament containing a bFGF-saporin conjugate is in theory self-targeting after intravenous injection to prevent restenosis following such surgical treatment or the like. Moreover, one (or a few) doses should prove sufficient, thus avoiding such potential problems as allergic reactions or production of neutralizing antibodies. The bFGF-saporin conjugate also has the useful feature of binding to heparin-like species on the cell surface, and if bFGF receptors are expressed later in some cells than others, the conjugate often remains in the region so as to be accessible once the receptor is expressed. The same advantage pertains if expression of the bFGF receptor is localized to only a portion of the cell cycle. A single treatment delivers the conjugate for temporary local storage, and because some cells internalize a small amount of heparin, FGF-SAP might be internalized even by cells not expressing FGF receptors. No obvious systemic toxicity is observed in normal animals receiving bFGF-saporin, and it may be that bFGF-SAP is cleared by binding of bFGF to alpha-2 macroglobulin, or shedding or degradation of cell surface heparin. Clearance might be accelerated by chasing with intravenous heparin, or with glutathione for reduction of bFGF-SAP disulfide bond.

-16-

Although local delivery of high concentrations of bFGF-SAP may be employed, the possibility of thrombosis indicates that intravenous administration, which did not produce necrosis or other obvious toxicity, is preferred. Because it appears that there are few FGF functional receptors in normal adult blood vessels, the toxicity of the bFGF-saporin conjugate should not have substantial undesirable side effects as a result of IV administration. Because replicating endothelial cells express FGF receptors, reendothelialization of vascular wounds can be delayed by bFGF-SAP administration; moreover, wound-healing, in general, may be delayed if fibroblasts and activated leukocytes express FGF receptors, or if their high levels of non-specific phagocytosis cause them to ingest saporin. Accordingly, after angioplasty, IV administration of bFGF-SAP may be delayed for a few weeks (to allow healing of the femoral artery access puncture), yet it should still be effective since restenosis apparently develops over several months.

For treatment of vascular injury, a therapeutically effective amount of a medicament containing an FGF-cytotoxic agent conjugate in a physiologically acceptable excipient is administered to a mammal. Examples of physiologically acceptable excipients include PBS and saline.

Preferably, the medicament containing the conjugate is administered intravenously (IV), although treatment by localized administration of the conjugate may be tolerated in some instances. Generally, the medicament containing the conjugate is injected into the circulatory system of a patient in order to deliver a dose of cytotoxin to the targeted cells by first binding the conjugate to high affinity bFGF receptors expressed by such cells.

The efficiency with which a cytotoxin, such as saporin or a Ricin A chain or a similar RIP, can inhibit

-17-

protein synthesis and consequently interfere with DNA synthesis is fairly widely known. Accordingly, the dosage of the conjugate that is administered will, to some extent, depend upon the particular cytotoxin chosen; however, doses of the conjugate in the range of about 0.01 mg to about 100 mg of the conjugate per kilogram of body weight are expected to be employed as a daily dosage.

Although the invention has been described with reference to the presently-preferred embodiments, it should be understood that various changes and modifications can be made without departing from the spirit of the invention, which is defined by the claims appended hereto.

15

-18-

WHAT IS CLAIMED IS:

1. Use of FGF conjugated to a cytotoxic agent for manufacture of a medicament for treatment of a patient who has suffered vascular injury.
2. The use of Claim 1 wherein said conjugate is formed from basic FGF.
3. The use of Claim 2 wherein said cytotoxic agent is a ribosome-inactivating protein.
4. The use of Claim 3 wherein said cytotoxic agent is saporin.
5. Use of FGF conjugated to a cytotoxic agent for manufacture of a medicament for treatment of a patient who has suffered an injury as a result of which there is occurring undesired proliferation of cells which express functional high-affinity bFGF receptors.
6. A method of preventing restenosis caused by smooth muscle cell proliferation in response to injury to the endothelial lining of blood vessel walls, which method comprises administering an effective amount of an FGF-conjugate sufficient to prevent smooth muscle cell proliferation in the lumen of the blood vessels, wherein said FGF is conjugated to a cytotoxic ribosome-inactivating protein.
7. The method of Claim 6 wherein said conjugate is formed from basic FGF.
8. The method of Claim 7 wherein said ribosome-inactivating protein is saporin.
9. The method of Claim 8 wherein said conjugate is administered intravenously in an amount sufficient to deliver between approximately .01 mg to 100 mg of conjugate per kilogram of body weight per day.
10. A method of preventing restenosis caused by smooth muscle cell proliferation in response to vascular surgery by balloon catheterization treatment or the like, which method comprises administering an

-19-

effective amount of FGF is conjugated to a cytotoxic ribosome-inactivating protein, said amount of FGF-conjugate being sufficient to prevent smooth muscle cell proliferation in the lumen of the blood vessels, said
5 administering occurring between 24 hours and six weeks after vascular surgery.

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 92/00101

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁹

According to International Patent Classification (IPC) or to both National Classification and IPC
 Int.C1.5 A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.C1.5	A 61 K 47/00 A 61 K 45/00

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ⁶	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9012597 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 01 November 1990, see abstract; claims 1,2,4,5,21; page 5, lines 3-11 ---	1-5
X	Biochemical and Biophysical Research Communications, Band 160, Nr. 2, 28 April 1989, D.A. LAPPI et al.: "Biological and chemical characterization of basic FGF-Saporin Mitotoxin", pages 917-923, especially page 917; abstract; page 922 ---	1-5
A	US,A,4950483 (G. KSANDER et al.) 21 August 1990, see abstract; claims 1-5 ---	1,5
A	EP,A,0305967 (CIBA-GEIGY AG) 8 March 1989, see abstract; claims 11,12; page 2, lines 9-25 -----	1,5

⁹ Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed


"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 04-05-1992	Date of Mailing of this International Search Report 27.05.92
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer  Danielle van der Haas

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 6-10 because they relate to subject matter not required to be searched by this Authority, namely:

(Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods)
Rule 39.1 (iv) PCT

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(e).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international searching authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANHANG

zum internationalen Recherchen-
bericht über die internationale
Patentanmeldung Nr.

ANNEX

to the International Search
Report to the International Patent
Application No.

ANNEXE

au rapport de recherche inter-
national relatif à la demande de brevet
international n°

PCT/US92/00101 SAE 56281

In diesem Anhang sind die Mitglieder
der Patentfamilien der in obenge-
nannten internationalen Recherchenbericht
angeführten Patentdokumente angegeben.
Diese Angaben dienen nur zur Unter-
richtung und erfolgen ohne Gewähr.

This Annex lists the patent family
members relating to the patent documents
cited in the above-mentioned inter-
national search report. The Office is
in no way liable for these particulars
which are given merely for the purpose
of information.

La présente annexe indique les
membres de la famille de brevets
relatifs aux documents de brevets cités
dans le rapport de recherche inter-
national visé ci-dessus. Les renseigne-
ments fournis sont donnés à titre indica-
tif et n'engagent pas la responsabilité
de l'Office.

In Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 9012597	01-11-90	EP A1 470183	12-02-92
US A 4950483	21-08-90	AU A1 39646/89	23-01-90
		EP A1 428541	29-05-91
		JP T2 4500954	20-02-92
		US A 5024841	18-06-91
		WO A1 9000060	11-01-90
EP A2 305967	08-03-89	AU A1 21725/88	23-03-89
		DK A0 4865/88	01-09-88
		DK A 4865/88	03-03-89
		EP A3 305967	31-01-90
		FI A0 884013	31-08-88
		FI A 884013	03-03-89
		IL A0 87621	31-01-89
		JP A2 1090200	06-04-89
		NO A0 883896	01-09-88
		NO A 883896	03-03-89
		NZ A 226005	26-10-90
		PT A 88385	31-07-89
		ZA A 8806471	26-04-89

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.